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(HC-Pro) Eliminate Potato Virus X/Potyviral Synergism

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Coinfection of tobacco plants with potato virus X (PVX) and any of several members of the potyvirus group causes a synergistic disease characterized by a dramatic increase in symptom severity correlated with a 3- to 10-fold increase in the accumulation of PVX in the first systemically infected leaves. We have recently shown that PVX/potyviral synergistic disease is mediated by expression of potyviral 5′-proximal sequences encoding P1, helper component-proteinase (HC-Pro), and a fraction of P3 (termed P1/HC-Pro sequence). Here we report the effect of mutations in this potyviral sequence on the induction of synergistic disease. Three transgenic tobacco lines expressing the tobacco etch potyvirus (TEV) P1/HC-Pro sequence with mutations within the P1 coding region were not impaired in their ability to mediate synergism when infected with PVX. In contrast, two of three transgenic lines with mutations in the HC-Pro coding region were unable to induce the synergistic increases in either symptom severity or PVX accumulation. Loss of synergistic function was associated with mutations within the region encoding the central domain of HC-Pro, while the ability to induce synergism was retained in a transgenic line expressing HC-Pro with an alteration in the amino-terminal "zinc-finger domain." In coinoculation experiments, a TEV mutant lacking the sequence encoding the zinc-finger domain of HC-Pro induced a typical synergistic response in interaction with PVX. The results indicate that the zinc-finger domain comprising the first 66 amino acid residues of HC-Pro is dispensable for induction of synergistic disease and transactivation of PVX multiplication, while regions within the central domain of HC-Pro are essential for both of these responses.

INTRODUCTION

Higher plants are commonly subject to mixed viral infections, and in many cases the dual viral infection results in disease symptoms more severe than the sum of those induced in either single infection. Many such synergistic diseases involve a member of the genus *Potyvirus* as one of the coinfecting viruses (for example, Anjos *et al.,* 1992; Clark *et al.,* 1980; Khan and Demski, 1982; Poolpol and Inouye, 1986). The best studied examples of potyvirus-associated synergism are caused by the interaction of PVX with any of several potyviruses, including potato virus Y (PVY), tobacco vein mottling virus (TVMV), and tobacco etch virus (TEV), and occur in tobacco and a variety of solanaceous plants (Damirdagh and Ross, 1967; Rochow and Ross, 1955; Vance, 1991; Vance *et al.,* 1995). Several other potyvirus-associated synergisms have been examined in some detail and these include corn lethal necrosis which is caused by the interaction of maize chlorotic mottle virus (MCMV) and maize dwarf potyvirus (Goldberg and Brakke, 1987), and soybean top necrosis which is caused by the interaction of bean pod mottle virus (BPMV) and soybean mosaic potyvirus (Lee and Ross, 1972; Calvert and Ghabrial, 1983). In all of these potyvirus-associated synergisms, the level of the nonpotyvirus in the viral pair (PVX, MCMV or BPMV) increases 3- to 10-fold in systemically infected leaves of doubly infected plants, while the level of the potyvirus in the same leaves is unchanged from that seen in singly infected plants. In each case, the increase in accumulation of the nonpotyvirus is correlated with the increased symptom severity typical of the synergistic disease.

We have recently identified the region of the potyviral genome that mediates the increases in PVX accumulation and pathogenicity characteristic of PVX/potyviral synergistic disease (Vance *et al.,* 1995). The region comprises the 5′ proximal portion of the genome, encoding the P1 and HC-Pro polypeptides and at least a part of the P3 polypeptide (termed P1/HC-Pro sequence). This region of the potyviral genome is initially expressed as a polyprotein that is subsequently cleaved to produce the mature viral proteins by the autoproteolytic activities of both P1 and HC-Pro (Carrington *et al.,* 1989; Verchot and Carrington, 1991). The effect of this potyviral sequence on PVX accumulation and pathogenicity may reflect the activities of the viral RNA, the encoded polyprotein, one or both mature viral proteins, or some combination of these factors. In this study we have used

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mutations to identify the elements within this region of the potyviral genome that are required to induce a synergistic response to PVX infection. We find that mutations within the region encoding the central domain of HC-Pro eliminate synergism. However, the amino-terminal region of HC-Pro containing a zinc-finger-like motif is not required.

METHODS

Viruses and plants

The virus strains used in these experiments were as follows: PVX (PV197, American Type Culture Collection) and a modified form of the highly aphid transmissible strain of TEV (TEV-HAT), the genome of which is represented as cDNA in the infectious clone pTEV7DA (Dolja *et al.,* 1992). TEV-2del is a spontaneous deletion mutant of a TEV vector that was constructed using the infectious pTEV7DA cDNA and has been previously described (Dolja *et al.,* 1993).

The six independent transgenic tobacco lines used in these studies were prepared by *Agrobacterium tumifaciens*-mediated transformation techniques as previously described (Verchot and Carrington, 1995). Construction of the transgenic line referred to here as TEV-B has been previously described (line 0027-1031-1B5, Verchot and Carrington, 1995), and the same basic protocol was used to generate the other five lines. Briefly, all lines were generated by introducing TEV sequence from nucleotides 12 to 2681 but with insertions of nine nucleotides at various regions within the P1 (TEV mutants B, C, and E) or HC-Pro (TEV mutants I, K, and L) coding sequences. The nine nucleotide insertion introduced an *Nco*I site into the mutant transgene DNA and resulted in insertion of the amino acid triplet Thr-Met-Ala immediately after amino acid 157 (TEV B), 188 (TEV C), 247 (TEV-E), 366 (TEV I), 426 (TEV-K), or 456 (TEV-L) of the expressed TEV polyprotein. Insertions were introduced into the TEV sequence by site-directed mutagenesis as previously described (Verchot *et al.,* 1991). TEV sequences were first cloned into the intermediate vector pRTL2 (Carrington *et al.,* 1990) and then transferred along with the cauliflower mosaic virus 35S promoter and terminator sequences to the binary vector pGA482 (An, 1987). Recombinant plasmids were introduced into *A. tumefaciens* LBA4404 and transgenic lines produced in *Nicotiana tabacum* cv Xanthi nc. All transgenic plants for the experiments described here were grown from seed germinated in the presence of kanamycin at a concentration of 600 μg/ml.

Inoculation and sampling

In experiments using transgenic plants, transgenic seeds were germinated and grown in an insect-free growth chamber and chosen to be the same size and in the same stage of development when inoculated with PVX (0.5 mg/ml purified virus). In each experiment, transgenic plants and control plants of the same variety of tobacco transformed with vector sequence (vector-only transformed plants) were mechanically inoculated on a single lower leaf with PVX. At least three plants from each transgenic line were observed for symptom development and assayed for levels of PVX RNA and coat protein, and the results were compared directly to those from vector-only-transformed control plants infected in the same experiment.

In coinoculation experiments, inoculation and sampling procedures of doubly and singly infected nontransgenic plants were as previously described (Vance, 1991) except that the inocula for both TEV-HAT and TEV-2del were prepared by grinding virus-infected leaves. TEV-2del-infected leaves were prepared by direct inoculation of Carborundum-dusted plants with infectious transcripts as previously described (Dolja *et al.,* 1993). In each of three experiments involving TEV-2del, one set of five nontransgenic tobacco plants (*N. tabacum* cv Xanthi nc) was mock inoculated or inoculated with PVX alone (0.5 mg/ml purified virus), TEV-HAT (undiluted extract), TEV-2del (undiluted extract), both PVX and TEV-HAT or both PVX and TEV-2del. The course of symptom development was as previously described for PVX/PVY synergistic disease (Vance, 1991), with doubly infected plants showing symptoms of acute synergistic disease on the first systemically infected leaves and much less dramatic symptoms on leaves invaded later (chronic stage of synergism). Nucleic acid and protein samples were taken from the leaf three to five nodes above the inoculated leaf at 8 to 10 days postinoculation (acute stage of synergism). The sampled leaf showed mild vein clearing symptoms in plants infected with TEV-HAT, little or no symptoms in plants infected with either TEV-2del or PVX only, and severe synergistic symptoms in plants coinfected with PVX and either TEV-HAT or TEV-2del. Equivalent portions of that leaf, not including the midvein, were used to isolate total nucleic acid. Portions of the opposite half-leaf were used to isolate protein. Nucleic acid and protein samples were assayed for levels of PVX (+) strand RNA and coat protein as described under Northern and Western analyses.

Northern and Western analyses

Relative levels of PVX genomic RNA were assayed by Northern analysis in side by side experiments with doubly and singly infected plants or with TEV transgenic and vector-only transformed plants. Total nucleic acids were isolated, separated by denaturing gel electrophoresis, and transferred to nylon membranes exactly as previously described (Vance, 1991). A radioactive cDNA probe complementary to the entire PVX (+) strand RNA

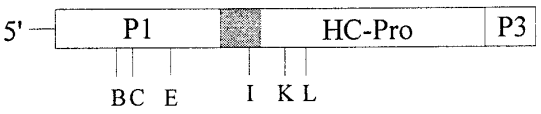
was generated using reverse transcriptase, hexanucleotide random primers, and purified PVX genomic RNA isolated from purified virus (Vance *et al.,* 1995). The levels of PVX RNA on Northern blots were quantitated by densitometric scanning of the X-ray film using a Microscan 1000 scanner (Technology Resource, Inc., Nashville, TN) as previously described (Vance *et al.,* 1995).

Levels of PVX coat protein or TEV wild-type or mutant HC-Pro were assayed by Western blot analysis. Total protein was extracted, quantitated, separated by SDS–PAGE as previously described (Vance, 1991), and transferred to PDVF paper (Bio-Rad Laboratories) using a Bio-Rad mini trans-blot apparatus at 80 V for 1 hr. Rabbit polyclonal antisera specific to either PVX coat protein (PV54a, American Type Culture Collection) or to TEV HC-Pro (Carrington *et al.,* 1990) were used as primary antisera and immunoreactive proteins visualized using a goat anti-rabbit antiserum conjugated to alkaline phosphatase (Bio-Rad Laboratories).

RESULTS

Transgenic plant experiments

We have previously shown that transgenic plants expressing the P1/HC-Pro region of the TEV genome respond dramatically to infection with PVX, showing the severe symptoms and increased accumulation of PVX that is typical of PVX/potyviral synergistic disease (Vance *et al.,* 1995). Here we use transgenic plants (*N. tabacum,* cv Xanthi nc) expressing mutant versions of this basic 2681 nucleotide construct to map specific regions of the TEV sequence which are important in the induction of synergy. The 5′-proximal 2681 nucleotide P1/HC-Pro region of the TEV genomic RNA contains 133 nucleotides of 5′-untranslated region and the coding regions for both P1 and HC-Pro polypeptides as well as approximately one fourth of the P3 polypeptide. The TEV polypeptides are initially expressed as a precursor polyprotein, which is subsequently processed by proteolytic activities of both P1 and HC-Pro. Six insertion mutations of this TEV sequence were constructed and tested initially for the level of transgene expression and then for ability to induce the synergistic response to PVX infection. Three of the six insertion mutations were within the P1 coding region (Verchot *et al.,* 1991) and three within the HC-Pro coding region, and the exact location of each of the resulting amino acid insertions within the basic construct is shown in Fig. 1. Western analysis with an antiserum specific to TEV HC-Pro was used to estimate the level of transgene expression and to assay for proteolytic processing of the P1/HC-Pro/P3 polyprotein in the six transgenic lines. Figure 2 shows that all six lines accumulated detectable levels of HC-Pro, in a range of approximately 10–50% of that seen in leaves of tobacco plants systemically infected with TEV (compare lanes 2–7 with lane 8). The lowest level of transgene expression, approximately 10% of that in a TEV infection, was detected in line B (Fig. 2, lane 2), and this expression level is consistent with that previously reported for that line (Cronin *et al.,* 1995). Line E expressed the highest levels of HC-Pro (Fig. 2, lane 4), while lines C, I, K, and L expressed at intermediate levels (Fig. 2, lanes 3, 5, 6, and 7, respectively). HC-Pro was processed correctly in each



TRANSGENE MUTANT	AA LOCATION	SYNERGISM
B	157 (P-1)	+
C	188 (P-1)	+
E	247 (P-1)	+
I	366 (HC-Pro)	+
K	426 (HC-Pro)	-
L	456 (HC-Pro)	-

FIG. 1. Diagram of the P1/HC-Pro region of the TEV genome showing the location of insertion mutations within the P1 and HC-Pro coding regions. The locations of the resulting three amino acid insertions within the mutant polyprotein and the abilities of the mutant transgenes to induce synergistic disease are indicated below the diagram. The HC-Pro coding sequence deleted from TEV-2del is indicated by stippling.

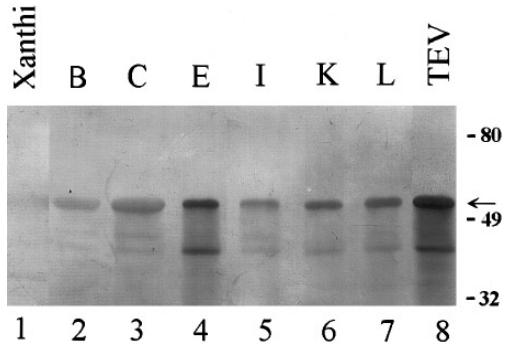


FIG. 2. Western analysis showing levels of TEV HC-Pro in TEV transgenic lines. Total protein isolated at Day 8 postinoculation from vector-only transformed control plants (lane 1), transgenic lines TEV-B (lane 2), TEV-C (lane 3), TEV-E (lane 4), TEV-I (lane 5), TEV K (lane 6), and TEV-L (lane 7) or TEV-infected plants (lane 8) was fractionated, electroblotted, probed with a polyclonal antiserum specific to TEV HC-Pro, and visualized using a secondary antiserum conjugated with alkaline phosphatase. Lanes 1, 2, and 3 have 60 μg of total protein loaded, while all the other lanes have 20 μg of total protein. The signal in lane 1 represents the background of nonspecific binding of the HC-Pro polyclonal antiserum to host proteins. The positions of protein molecular weight standards are shown by numbers (in kDa) to the right of the figure and the position of processed HC-Pro is indicated by an arrow.

FIG. 3. Symptoms displayed in plants of transgenic lines TEV-I (left), TEV-K (center), and TEV-L (right) systemically infected with PVX.

of the six mutant transgenic lines as assayed by comigration of the immunoreactive band with authentic HC-Pro from TEV-infected plants (compare lanes 2–7 with lane 8); correct processing of HC-Pro from the parental construct in transgenic line U-6B was previously reported (Carrington *et al.,* 1990).

To determine whether the mutant TEV sequences maintained the ability to induce synergism, plants from each transgenic line were inoculated with PVX, observed for symptoms, and assayed for levels of accumulation of PVX (+) strand RNA and PVX coat protein. Sets of plants from each of the six mutant lines and the vector transformed control line were mechanically inoculated with PVX as previously described (Vance, 1991). Assays were carried out using the first systemically infected leaves (leaf 3, 4, or 5 above the inoculated leaf) at Day 8 or Day 10 postinoculation, depending on the particular experiment. The strain of PVX used for these experiments produced few if any symptoms in *N. tabacum,* cv Xanthi plants. In contrast, transgenic plants expressing mutant versions of P1 displayed various levels of the initial severe vein clearing, mottling, and eventual necrosis typical of PVX/potyviral synergistic disease (data not shown). Similar synergistic symptoms developed in the first systemically infected leaves of line I, which carries an insertion within the amino-terminal region of HC-Pro, and led to the death of those leaves and the growing tip of the plant by Day 10 postinoculation (Fig. 3, transgenic line I, left plant). In contrast, the two transgenic lines expressing HC-Pro with insertions within the central domain of the protein remained essentially symptomless (Fig. 3, transgenic lines K and L, center and right plants, respectively). The failure of mutants K and L to induce the increase in PVX pathogenicity was not due to inadequate accumulation of the mutant HC-Pro, as the K and L mutant versions of HC-Pro appear to accumulate to levels at least as high as that of the mutant I plant, and higher than those of mutant B and C plants, all of which are capable of inducing synergism (Fig. 2, compare lanes 6 and 7 to lanes 2, 3, and 5). These results indicate that the TEV sequence encoding the central domain of HC-Pro is essential for induction of synergistic disease symptoms.

To determine whether mutant TEV sequences also maintain the ability to induce the known synergistic increase in accumulation of PVX, the levels of PVX genomic RNA and coat protein in the six mutant transgenic lines were compared to those in vector-only transformed control plants. RNA and protein were isolated from the leaf three nodes above the inoculated leaf, and the levels of PVX RNA and coat protein were assayed by Northern and Western blot analyses, respectively. Figure 4A shows that higher levels of PVX (+) strand RNA have accumulated in leaves of mutants B, C, E, and I relative to that detected in the PVX-infected vector-only transformed control plant (compare the signal in lanes 1–4 to that in lane 6, which is from an approximate 3.5-fold longer exposure of the same blot). The increase in the level of PVX genomic RNA in the different transgenic lines ranged from approximately 5- to 11-fold as determined by densitometric scanning of slot blots loaded with serial dilutions of total RNAs from acutely affected leaves (data not shown). In contrast, the levels of PVX RNA in leaves of mutants K and L were approximately equal to those

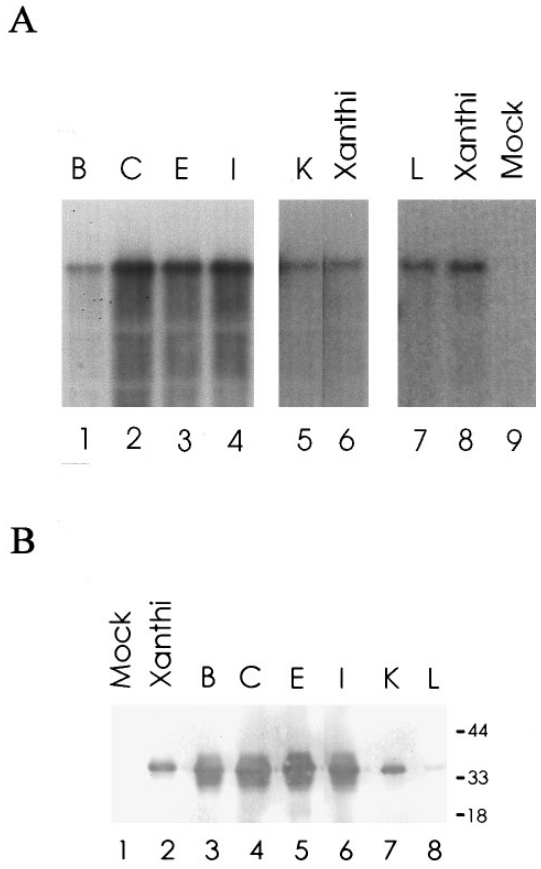


FIG. 4. Northern and Western analyses showing levels of PVX genomic RNA and PVX coat protein in leaves of transgenic tobacco plants systemically infected with PVX. (A) Total plant RNA (5 μg/lane) isolated from mock-inoculated tobacco plants (lane 9) or from PVX-infected TEV transgenic lines TEV-B (lane 1), TEV-C (lane 2), TEV-E (lane 3), TEV-I (lane 4), TEV-K (lane 5), TEV-L (lane 7), or from vector-only transformed plants (lanes 6 and 8). The RNA samples were subjected to denaturing gel electrophoresis, transferred to nylon paper, and hybridized with ^{32}P-labeled cDNA complementary to PVX genomic RNA. RNAs in lanes 1–6 are from one set of contemporaneous samples isolated in a single experiment and run on the same gel. Lanes 1–4 show a short exposure of the blot and lanes 5 and 6 are from an approximate 3.5-fold longer exposure of the same blot. The RNA samples in lanes 7–9 are from an independent experiment. (B) Total plant protein (0.5 μg/lane) isolated from mock-inoculated tobacco plants (lane 1) or from PVX-infected transgenic lines TEV-B (lane 3), TEV-C (lane 4), TEV-E (lane 5), TEV-I (lane 6), TEV-K (lane 7), or TEV-L (lane 8) or from vector-only transformed plants (lane 2) and fractionated, electroblotted, and probed with antiserum specific for PVX coat protein. The positions of protein molecular weight standards are indicated by numbers (in kDa) to the right of the figure.

detected in vector-transformed control plants (Fig. 4A, compare lanes 5 and 6 for line K results and lanes 7 and 8 for line L results). Analysis of the coat protein levels gave a similar result: higher levels of PVX coat protein in mutants B, C, E, and I than in control plants (Fig. 4B, compare lanes 3, 4, 5, and 6 to lane 2), but no similar increase in mutants K or L (Fig. 4B, compare lanes 7 and 8 to lane 2). In fact, the level of coat protein accumulation in mutant L plants was consistently lower than that in control plants. These results indicate that the region of the TEV genome encoding the central domain of HC-Pro is required, not only for the increase in PVX pathogenicity, but also for the increase in PVX accumulation characteristic of PVX/potyviral synergism.

Mutant TEV experiments

Our results with transgenic plants indicated that the region of the TEV genome encoding the central domain of HC-Pro is required for synergism, while that encoding the amino-terminus may be dispensable. To further test whether the 5′ end of the HC-Pro coding sequence is required for synergism, we carried out coinoculation experiments with PVX and a TEV mutant lacking the genomic sequence that encodes the amino-terminal region of HC-Pro. The TEV mutant tested, TEV-2del (Dolja *et al.,* 1993), is a spontaneous deletion of a virus engineered to express bacterial β-glucuronidase (GUS) fused to HC-Pro. It lacks the coding region for 66 amino acid residues of the HC-Pro amino-terminus and carries 45 amino acid residues derived from the amino-terminus of GUS. The TEV-2del mutant has been shown to be viable in tobacco plants and can spread systemically, with virus accumulating in systemically infected leaves to a level approximately 17–25% of that in wild-type TEV infections, as estimated from levels of genomic RNA and coat protein in systemically infected leaves (Dolja *et al.,* 1993). Under the standard inoculation and growth conditions used for our PVX/TEV synergism experiments, the TEV-2del mutant-infected *N. tabacum* and spread systemically with about the same timing as the wild-type TEV-HAT, producing a very mild vein clearing and mottling in leaves three or more above the inoculated leaf. Western analysis using antiserum specific to HC-Pro indicated that systemic infection with TEV-2del resulted in accumulation of an immunoreactive band (Fig. 5, lane 1) that migrated slightly faster than wild-type TEV HC-Pro (Fig. 5, lane 2). HC-Pro accumulation in leaves systemically infected with TEV-2del was only slightly less than that detected in leaves infected with the wild-type virus (Fig. 5). The accumulation of wild type and mutant HC-Pro proteins was not altered by coinfection of the plants with PVX (data not shown).

Plants coinoculated with PVX and TEV-2del developed the severe symptoms typical of PVX/potyviral synergistic disease (data not shown). To determine if coinfection with TEV-2del also induced an increase in PVX accumulation, the relative levels of PVX genomic RNA and coat protein in the first systemically infected leaves of doubly and singly infected plants were determined by Northern and Western analyses, respectively. The level of PVX genomic RNA was about 7-fold greater in plants doubly infected with PVX and either wild type TEV-HAT (Fig. 6A,

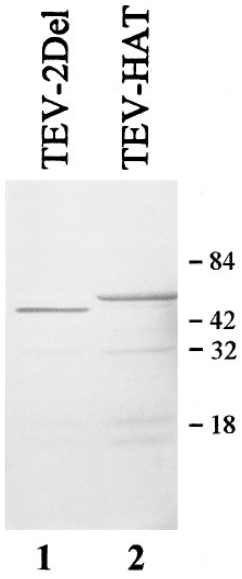


FIG. 5. Western analysis showing levels of wild-type TEV HC-Pro or a deleted version of the protein in tobacco plants systemically infected with either TEV-HAT (lane 2) or TEV-2del (lane 1). Total protein was isolated from the third leaf above the inoculated leaf of the infected plants at Day 8 postinoculation, fractionated by gel electrophoresis (20 μg/lane), and analyzed as described in the legend of Fig. 2. The positions of protein molecular weight standards are indicated by numbers (in kDa) to the right of the figure.

lane 2) or TEV-2del (Fig. 6A, lane 3) than in plants infected with PVX alone (Fig. 6A, lane 1). Western analysis showed a similar increase in PVX coat protein in plants doubly infected with PVX and either TEV-HAT (Fig. 6B, lane 2) or TEV-2del (Fig. 6B, lane 3) over that in plants infected with PVX alone (Fig. 6B, lane 1). These results indicate that the region of the TEV genomic RNA encoding the amino-terminal 66 amino acid residues of HC-Pro is dispensable for induction of synergistic disease and transactivation of PVX multiplication.

DISCUSSION

Synergistic viral diseases in plants have been known for a long time, and elucidating the mechanisms by which coinfecting viruses act to enhance pathogenesis is a classic problem in plant pathology. The PVX/TEV synergism is a good model system to investigate the underlying molecular mechanisms of synergistic disease because it is the best studied of the plant viral synergisms and is typical of many such synergistic diseases. Our previous work has shown that expression of the TEV P1/HC-Pro sequence is sufficient to induce the synergistic enhancement of PVX accumulation and pathogenicity; however, it was not known whether the entire region is necessary for induction of synergistic disease. The work presented here indicates that the potyviral sequence encoding the central domain of HC-Pro is required in the induction of synergistic disease. In contrast, the region encoding the zinc-finger domain at the amino-terminus of HC-Pro is dispensable for this process. Our experiments failed to identify a domain of P1 that is required for induction of synergism, since none of the mutations within the P1 coding region that were tested affected the ability of the sequence to induce synergism. However, these results do not rule out a role for P1 in the synergistic response and further experimentation is needed to define the minimal potyviral sequence required for the synergistic interaction with PVX.

Like many viral proteins, HC-Pro is a multifunctional protein, playing roles in aphid transmission of the potyvirus (Berger *et al.,* 1989; Thornbury *et al.,* 1990), proteolytic processing of the viral polyprotein (Carrington *et al.,* 1989), genome amplification and pathogenicity (Atreya *et al.,* 1992; Atreya and Pirone, 1993; Dolja *et al.,* 1993; Kasschau and Carrington, 1995; Kasschau *et al.,* 1997; Klein *et al.,* 1994; Thornbury *et al.,* 1990), and long distance transport of the virus (Cronin *et al.,* 1995; Kasschau *et al.,* 1997). Our findings add a new HC-Pro function to what appears to be an ever-growing list: induction of synergistic increases in the accumulation and pathogenicity of the heterologous plant virus PVX. The amino-terminal 66 amino acid residues of HC-Pro are required for aphid transmission but not for long-distance move-

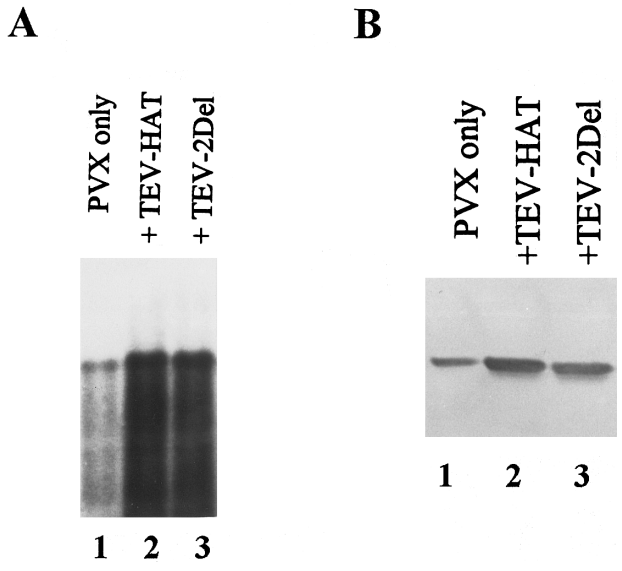


FIG. 6. Northern and Western analyses showing levels of PVX genomic RNA and PVX coat protein in leaves of tobacco plants systemically infected with PVX or doubly infected with PVX and either TEV-HAT or TEV-2del. (A) Total plant RNA isolated from the third leaf above the inoculated leaf of tobacco plants at Day 8 after inoculation with PVX alone (lane 1) or coinoculation with PVX and either TEV-HAT (lane 2) or TEV-2del (lane 3). The RNA samples were fractionated (5 μg/lane), blotted, and hybridized as described in the legend of Fig. 4. (B) Total plant protein isolated from the third leaf above the inoculated leaf of tobacco plants at Day 8 after inoculation with PVX alone (lane 1) or coinoculation with PVX and either TEV-HAT (lanes 2) or TEV-del2 (lane 3).

ment of the potyvirus in the phloem of the plant. We find that the sequence encoding this region of HC-Pro is also dispensable for its role in synergism. In contrast, the central domain of HC-Pro, which has been implicated in pathogenicity, genome amplification, and long-distance movement of the potyvirus in plants, is essential for induction of synergism.

The mechanism by which potyviral sequences mediate synergistic disease is not yet clear. One hypothesis is that potyviral proteins affect PVX accumulation directly by binding to the PVX RNA or the viral replication complex and altering a key step in RNA replication. The P1 protein of TVMV has been shown to bind nucleic acids, preferentially single-stranded RNA (Brantley and Hunt, 1993). Likewise, HC-Pro is known to bind both ribopolymers (Thornbury *et al.,* 1985) and RNA (Maia and Bernardi, 1996), again with a preference for single-stranded RNA. In the case of HC-Pro, the zinc-finger domain at the amino-terminal region of the protein has been speculated to be involved in both dimerization of the protein and in nucleic acid binding (Atreya *et al.,* 1992; Thornbury *et al.,* 1985). Our results indicate that the zinc finger domain of HC-Pro is dispensable for synergism, and therefore raise the possibility that the ability of HC-Pro to dimerize and bind nucleic acid may not be a requirement for induction of synergistic disease.

Two lines of evidence suggest that the synergistic increase in PVX accumulation is due to enhanced replication. First, the proportion of PVX-infected cells in the first systemically infected leaves of doubly infected plants displaying synergistic disease is the same as that in plants infected singly with PVX (Goodman and Ross, 1974). This result indicates that the increase in PVX accumulation in doubly infected leaves is due to an increase in the level of virus per cell rather than an increase in the number of infected cells per leaf. Second, the level of PVX (−) strand RNA, an intermediate in PVX RNA replication, is increased significantly more than that of PVX (+) strand RNA during synergism, suggesting an alteration in the regulation of RNA replication (Vance, 1991). However, since infection of whole plants is asynchronous and involves both replication and movement of the virus, neither of these lines of evidence rule out the alternative hypothesis that the increased accumulation of PVX in synergism is due entirely to an increase in the efficiency of viral movement through the plant. A third possibility is that the potyviral sequence affects both the replication and the movement of PVX and this hypothesis is strengthened by the observation that the central domain of HC-Pro, which plays a role in both replication and movement of the potyvirus (Kasschau *et al.,* 1997), is also required for its role in synergism. This observation also raises the possibility of sorting out the respective roles of replication and movement in synergism using HC-Pro mutants with effects on only one process or the other. The mechanism by which HC-Pro influences potyviral replication, pathogenicity, and long-distance movement in the phloem is not known, but the protein may well function in an analogous fashion to enhance the replication and/or movement of PVX during synergistic infections. Thus information gained in one system may lead to progress in understanding the other.

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